Amplicon mapping and expression profiling identify the fas-associated death domain gene as a new driver in the 11q13.3 amplicon in Laryngeal/Pharyngeal cancer

Gibcus, J.H.; Menkema, L.; Mastik, M.F.; Hermsen, M.A.; de Bock, G.H.; van Velthuysen, M.L.; Takes, R.P.; Kok, K.; Alvarez Marcos, C.A.; van der Laan, B.F.

Published in:
Clinical Cancer Research

DOI:
10.1158/1078-0432.CCR-07-1247

Publication date:
2007

Amplicon Mapping and Expression Profiling Identify the Fas-Associated Death Domain Gene as a New Driver in the 11q13.3 Amplicon in Laryngeal/Pharyngeal Cancer

Johan H. Gibcus,[1] Lorian Menkema,[1] Mirjam F. Mastik,[1] Mario A. Hermsen,[6] Geertruida H. de Bock,[2] Marie-Louise F. van Velthuysen,[7] Robert P. Takes,[9] Klaas Kok,[5] Cesar A. Álvarez Marcos,[6] Bernard F.A.M. van der Laan,[3] Michiel W.M. van den Brekel,[8] Johannes A. Langendijk,[4] Philip M. Kluin,[1] Jacqueline E. van der Wal,[1] and Ed Schuuring[1]

Abstract

Purpose: Amplification of the 11q13 region is a frequent event in human cancer. The highest incidence (36%) is found in head and neck squamous cell carcinomas. Recently, we reported that the amplicon size in 30 laryngeal and pharyngeal carcinomas with 11q13 amplification is determined by unique genomic structures, resulting in the amplification of a set of genes rather than a single gene.

Experimental Design: To investigate which gene(s) drive the 11q13 amplicon, we determined the smallest region of overlap with amplification and the expression levels of all genes within this amplicon.

Results: Using array-based comparative genomic hybridization analysis, we detected a region of ~1.7 Mb containing 13 amplified genes in more than 25 of the 29 carcinomas. Quantitative reverse transcription-PCR revealed that overexpression of 8 potential driver genes including, *cyclin D1, cortactin*, and Fas-associated death domain (*FADD*), correlated significantly with DNA amplification. FADD protein levels correlated well with DNA amplification, implicating that FADD is also a candidate driver gene in the 11q13 amplicon. Analysis of 167 laryngeal carcinomas showed that increased expression of FADD ($P = 0.007$) and Ser^{194} phosphorylated FADD ($P = 0.011$) were associated with a worse disease-specific survival. FADD was recently reported to be involved in cell cycle regulation, and cancer cells expressing high levels of the Ser^{194} phosphorylated isoform of FADD proved to be more sensitive to Taxol-induced cell cycle arrest.

Conclusion: Because of the frequent amplification of the 11q13 region and concomitant overexpression of FADD in head and neck squamous cell carcinomas, we hypothesize that FADD is a marker to select patients that might benefit from Taxol-based chemoradiotherapy.

Authors' Affiliations: Departments of [1]Pathology, [2]Epidemiology and Statistics, [3]Otorhinolaryngology, Head and Neck Surgery, [4]Radiation Oncology, and [5]Human Genetics, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands; [6]Department of Otolaryngology, Instituto Universitario de Oncología del Principado de Asturias, Oviedo, Spain; Departments of [7]Pathology and [8]Head and Neck Oncology and Surgery, the Netherlands Cancer Institute-Antoni Van Leeuwenhoek Hospital, Amsterdam, the Netherlands; and [9]Department of Otolaryngology/Head and Neck Surgery, Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands

Received 5/21/07; revised 7/2/07; accepted 7/25/07.

Grant support: De Drie Lichten, the Maurits and Anna de Kock Foundation, the Jan Kornelis de Cock Stichting, the Groningen University Institute for Drug Exploration, and Fondos de Investigación Sanitaria grant PI02-0831.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Requests for reprints: Ed Schuuring, Department of Pathology, University Medical Center Groningen, P.O. Box 30001, 9700 RB, Groningen, the Netherlands. Phone: 31-50-361-9623; Fax: 31-50-361-9107; E-mail: e.schuuring@path.umcg.nl.

doi:10.1158/1078-0432.CCR-07-1247

Squamous cell carcinoma of the head and neck (HNSCC) is the fifth most common cancer with a worldwide incidence of ~780,000 new cases per year. Traditionally, depending on tumor stage, patients are treated with surgery, radiotherapy alone, or a combination of both. In early to intermediate stages of laryngeal carcinoma, radiotherapy is generally preferred because of better organ preservation and high rates of locoregional tumor control. However, in the more advanced carcinomas, radiotherapy fails in local control in up to 50% of patients (1). A recent clinical trial showed that concomitant chemotherapy and radiotherapy leads to improved locoregional control and laryngeal preservation in locally advanced laryngeal cancer as compared with conventional radiotherapy (2, 3). However, combining chemotherapy with radiotherapy also has unfavorable effects because it results in a significant increase in toxicity. Although various clinicopathologic variables have been validated to classify patients according to their likelihood of responding to chemoradiation, they are still generally of low predictive value (1). Therefore, there is a need for (molecular) markers to predict therapeutic outcome.

Chromosomal DNA amplification resulting in an increase in gene copy number is a well-documented mechanism for cells to increase gene expression and has been used successfully as a prognostic marker for therapeutic outcome (4, 5). DNA amplification has been reported in various human malignancies and numerous (candidate) oncogenes have been identified. Amplification of chromosome 11q13 is frequently found in human cancer and is most prominent (36%) in HNSCC (6). Within different cancer subtypes, this amplification is associated with poor prognosis (reviewed in refs. 6, 7). These studies report HNSCC to be associated with the presence of lymph node metastasis and decreased disease-free survival and overall survival (8–11). In the last 15 years, many efforts were undertaken to identify the gene that is responsible for the biological behavior of the carcinomas with 11q13 amplification (6, 7). We and others have identified several candidate genes in the amplified 11q13 region that showed increased expression on amplification, including *CCND1* (cyclin D1; refs. 12, 13), *CTTN* (EMS1/cortactin; ref. 13), *MYEOV* (14), *ORAOV1* (TAOS1; ref. 15), *EMSY* (16), and *SHANK2* (17). *CCND1* and *CTTN* were the genes identified first and studied most extensively, and expression of both genes has been correlated with prognosis (reviewed in refs. 6, 7). The functions of cyclin D1, which is involved in cell cycle regulation, and cortactin, a regulator for actin-polymerization and cell migration and invasion (18–20), make them both good candidates of being the drivers of 11q13 amplification. However, the function and role of the other amplified and overexpressed 11q13 genes are presently unknown. Furthermore, most studies fail in comprehensively studying both the copy number and expression of all genes present in the amplicon.

The completion of the human genome sequence has enabled the use of new techniques like array-based comparative genomic hybridization (aCGH) to study genomic aberrations in more detail (4, 21, 22). The use of fluorescent probes in PCR analysis can be used to study gene expression [quantitative reverse transcription-PCR (RT-PCR)] or microsatellites [quantitative microsatellite analysis (QuMA)] quantitatively. These techniques have previously been used successfully to identify possible target genes in an amplicon (23, 24).

We generated a high-resolution aCGH specific for the long arm of chromosome 11 (25). In this article, we used these data to determine the core region of amplification (CRA). Second, we used quantitative RT-PCR for genes located in the CRA to search for candidate genes with a high relative expression. We found that not a single gene but 8 of the 13 genes in the CRA were overexpressed in at least 25 of 29 laryngeal/pharyngeal carcinomas with 11q13 amplification. In addition to cyclin D1, cortactin, and ORAOV1, this is the first study reporting on Fas-associated death domain (FADD) as a potential driver in the 11q13 amplicon. The possible role of *FADD* in the prognosis of laryngeal HNSCC was further studied immunohistochemically with antibodies against FADD protein and its phosphorylated isoform (pFADD) on a large subset of patients.

Materials and Methods

Patient material. For high-resolution aCGH analysis, we used snap-frozen tissues of squamous cell carcinomas from 42 patients. Selected carcinomas with a known 11q13 aberration originated from the larynx (n = 19) or pharynx (n = 11). The remaining 12 patients without an 11q13 aberration were diagnosed with laryngeal carcinomas. Nineteen patients (obtained from the Instituto Universitario de Oncología del Principado de Asturias, Oviedo, Spain) diagnosed with a laryngeal (L13-L23) or pharyngeal (P01-P08) carcinoma were previously screened for 11q13 abnormalities using conventional CGH (26). Five patients (L09-L12 and A11; from the Leiden University Medical Center, Leiden, the Netherlands) were included because of previous 11q13 amplification detected by Southern blotting (27). Eighteen patients with laryngeal (n = 15) or pharyngeal cancer (n = 3) were obtained from the University Medical Center Groningen, the Netherlands. These patients were prescreened with whole-genome aCGH (25) for the presence (L03-L07 and P09-P11) or absence (A01-A10) of 11q13 aberrations. For quantitative RT-PCR, only laryngeal carcinomas were included (L01-L08 and A01-A10). The percentage of tumor cells in the frozen sections used for DNA and RNA isolation was estimated by H&E staining judged by an experienced pathologist (J.E.vdW). All patient samples were primary tumors that had received no therapy before surgery. DNA from these patients was isolated by a standard high-salt extraction method.

A total of 167 laryngeal squamous cell carcinomas were used for immunohistochemical analysis, including material from the laryngeal carcinomas that were also used for high-resolution aCGH analysis (L01-L23). Patient material and available clinicopathologic data were obtained from the University Medical Center Groningen (n = 56); the Netherlands Cancer Institute-Antoni Van Leeuwenhoek Hospital, Amsterdam, the Netherlands (n = 61); the Instituto Universitario de Oncología del Principado de Asturias (n = 22); and the Leiden University Medical Center (n = 28). Patients were diagnosed at a median age of 61 years (minimum, 34 years; maximum, 89 years) with a gender distribution of 135 (88%) males and 19 (12%) females. T status at diagnoses was subdivided into T_1 (n = 16; 10%), T_2 (n = 27; 17%), T_3 (n = 38; 24%), and T_4 (n = 75; 48%) according to the American Joint Committee on Cancer TNM system. At the time of diagnosis, 63 (41%) patients were lymph node positive (N_1, 23; N_2, 35; N_3, 5) and 92 (59%) were lymph node negative (N_0). Patients were treated with primary surgery (n = 54), primary radiotherapy (n = 24), or a combination of both modalities (n = 64). All patient samples were taken before treatment.

High-resolution aCGH. A detailed description of the generation of the high-resolution chromosome 11q13-specific aCGH and the determination of the size of the amplified region as well as the copy number of the 11q13 region in the same 30 carcinomas used in this study were recently described elsewhere (25).

Quantitative RT-PCR. RNA was extracted from snap-frozen tumor tissue or cell lines using Trizol (Invitrogen) and cDNA was prepared. Quantitative measurements of mRNA content were done using an ABI PRISM 7900HT and the SDS software 2.1 (Applied Biosystems). TaqMan primers and probes are listed in Supplementary Table S1. No appropriate primer/probe sets could be designed for the hypothetical genes *LOC390218*, *LOC399921*, and *LOC653621* (Supplementary Methods). No RNA-specific primer/probe sets could be designed for *MRGPRD* and *MRGPRF*. The relative copy number of each unknown sample (ΔC_t) was obtained by normalization to TATA-box binding protein (*TBP*). Patients were divided into two groups on a gene-by-gene basis. The first group consisted of patients with 11q13.3 amplification containing the gene of interest and the second group included patients without the particular 11q13.3 amplification as determined by high-resolution aCGH. For a detailed description of the quantitative RT-PCR, see Supplementary Methods.

Immunohistochemistry. Paraffin-embedded, formalin-fixed sections of laryngeal carcinoma were deparaffinized and antigen retrieval was done by overnight incubation at 80°C in Tris-HCl pH 9.0 (for FADD) or heating in a microwave oven for 15 min in EDTA pH 8.0 (for Ser^{194} phosphorylated FADD and cleaved caspase-3). After blocking endogenous peroxidases with 0.3% H_2O_2, the sections were stained for 1 h with a mouse immunoglobulin G antibody against FADD (clone A66-2; 1:100; BD PharMingen) or caspase-3 (Asp175; 1:50; Cell Signaling) or

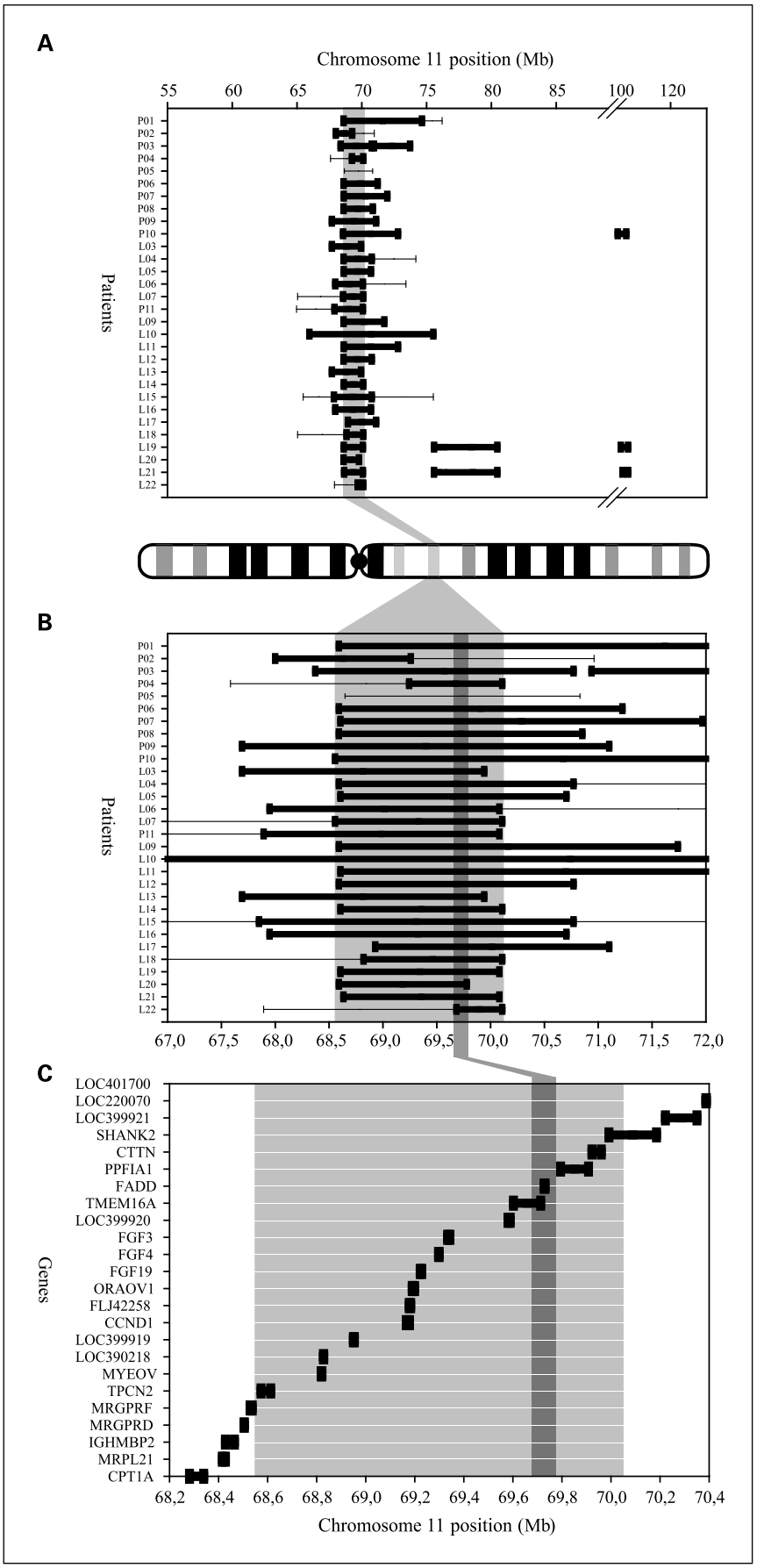

Fig. 1. Detailed location of the CRA at 11q13.3 as identified with 30 patients. Mapping of the amplified 11q13 regions in laryngeal carcinomas using a high-resolution chromosome 11q13 aCGH as previously reported (27). *A,* for each patient, the log 2 ratios exceeding 0.5 that were detected by aCGH smooth (29) are depicted as thick bars; detected gains (ratio below 0.5) are depicted as thin bars; normal ratios and deletions (no gain or amplification) are not included. *B,* detailed view with the CRA shown by a gray background. The gray region illustrates part of the 11q13.3 region amplified in at least 25 of 29 cases. The highest overlap was found at the dark gray region (amplified in 28 of 29 cases). *C,* the genes localized in the CRA are visualized as bars according to their location in megabases (Mb) on chromosome 11 (NCBI build 35.1).

stained overnight with an antibody against Ser^{194} phosphorylated FADD (1:25; Cell Signaling Technologies) diluted in 1% bovine serum albumin-PBS. Secondary and tertiary antibodies were diluted 1:100 in 1% bovine serum albumin-PBS complemented with 1% human AB serum or EnVision (DAKO). Antibodies were precipitated with 3,3′-diaminobenzidine tetrachloride as a substrate and the slides were counterstained with routine hematoxylin treatment. Immunohistochemistry for active caspase-3 was done on a specific subset of tumors with a high or low FADD expression.

Statistics. Statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS) version 14.0.0. To determine which factors were associated with the presence of FADD or Ser^{194} phosphorylated FADD protein, odds ratios were estimated by univariate and multivariate logistic regression with the presence of FADD or expression as dependent variable. To explore the effect of FADD or Ser^{194} phosphorylated FADD expression on the prognosis, Kaplan-Meier curves for overall survival (dead or alive) and disease-specific survival (dead of disease against alive or dead by other causes) were constructed and log-rank tests were done. To assess whether the effect of FADD or Ser^{194} phosphorylated FADD was independent of other well-known predictors, a forward-step multivariate Cox regression analysis was done including the variables that contributed statistically significantly in the univariate Cox regression analysis. A model including FADD as well as a model including Ser^{194} phosphorylated FADD was done. All tests were two sided and $P < 0.05$ was considered significant.

Results

The commonly amplified 11q13.3 region is restricted to 1.7 Mb and contains 13 genes. Using high-resolution aCGH, we recently showed gain ($n = 1$) and amplifications ($n = 29$) in all carcinomas with previously identified 11q13 amplifications, whereas normal copies were shown in all cases without previously identified 11q13 amplifications (25). The amplified region in all 29 cases was located within the same 5-Mb region at 11q13.3 (position 67-72 Mb; NCBI Build 35; summarized in Fig. 1A). *EMSY* (position 75.8 Mb), recently identified as a BRCA2-inactivating protein, which is amplified independent of *CCND1* in breast cancer (16), was amplified only in two cases (L19 and L21). The 11q22 gene cluster containing matrix metallopeptidases and the inhibitors of apoptosis *cIAP2* and *cIAP3* was amplified in 3 of our 29 carcinomas (P10, L19, and L21). This finding is in agreement with a previous report in esophagus cell lines (4 of 42 cases; ref. 28).

Within the 5-Mb commonly amplified region, we defined CRA-containing amplifications in at least 25 of the 29 cases (gray region in Fig. 1B). This region is flanked by genes that are coamplified at lower frequency (<18 of 29; Table 1). The CRA extends from *TPCN2* to *SHANK2* and is ~1.7 Mb in size (68.5-70.2 Mbp; build 35) containing 13 genes including

Table 1. Genes located at the 11q13.3 amplicon

#Start	Stop	Gene	Gene ID	Ref seq	Description	Amplification	Expression
68281657	68339532	*CPT1A*	1374	NM_001876	Carnitine palmitoyltransferase 1A (liver)	8 of 29	0.002
68415323	68427879	*MRPL21*	219927	NM_181512	Mitochondrial ribosomal protein L21	8 of 29	0.005
68427948	68464635	*IGHMBP2*	3508	NM_002180	Immunoglobulin μ binding protein 2	8 of 29	0.014
68504066	68505031	*MRGPRD*	116512	NM_198923	MAS-related GPR, member D	8 of 29	nd
68528443	68537311	*MRGPRF*	219928	NM_145015	MAS-related GPR, member F	18 of 29	nd
68572941	68612568	*TPCN2*	219931	NM_139075	Two pore segment channel 2	25 of 29	0.0001
68818198	68821330	*MYEOV*	26579	NM_138768	Myeloma overexpressed gene (in a subset of t(11;14) positive multiple myelomas)	26 of 29	ns
68827449	68828818	*LOC390218*	390218	XM_497307	Similar to IFN-induced transmembrane protein 3 (1-8U); IFN-inducible	27 of 29	nd
68949639	68953570	*LOC399919*	399919	XM_378299	Hypothetical LOC399919	27 of 29	nd
69165054	69178423	*CCND1*	595	NM_053056	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	27 of 29	0.0043
69177025	69183303	*FLJ42258*	440049	NM_001004327	Hypothetical protein supported by AK124252, FLJ42258	27 of 29	0.0019
69189515	69199296	*ORAOV1*	220064	NM_153451	Oral cancer overexpressed 1, TAOS1	27 of 29	<0.0001
69222187	69228287	*FGF19*	9965	NM_005117	Fibroblast growth factor 19	27 of 29	0.019
69296978	69299352	*FGF4*	2249	NM_002007	Fibroblast growth factor 4 (heparin secretory transforming protein 1, Kaposi sarcoma oncogene)	27 of 29	ns
69333917	69343129	*FGF3*	2248	NM_005247	Fibroblast growth factor 3 (murine mammary tumor virus integration site (v-int-2) oncogene homologue)	27 of 29	ns
69579986	69589125	*LOC399920*	399920	XM_378300	Hypothetical LOC399920	27 of 29	nd
69602294	69713282	*TMEM16A*	55107	NM_018043	Transmembrane protein 16A, TAOS2	27 of 29	ns
69726917	69731144	*FADD*	8772	NM_003824	Fas (TNFRSF6)-associated via death domain	28 of 29	<0.0001
69794471	69908150	*PPFIA1*	8500	NM_003626	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), α1	27 of 29	<0.0001
69922292	69960338	*CTTN*	2017	NM_005231	Cortactin, EMS1	27 of 29	0.013
69991609	70185520	*SHANK2*	22941	NM_012309	SH3 and multiple ankyrin repeat domains 2	25 of 29	ns
70221832	70350510	*LOC399921*	399921	XM_374904	Similar to SH3 and multiple ankyrin repeat domains protein 2 (Shank2)	16 of 29	nd

NOTE: Genes are ordered by their position on the chromosome (start-stop; Build 35). The number of patients with amplification of each gene as determined by aCGH is shown in the column "Amplification" (29 cases tested). The column "Expression" shows the *P* value given by a Mann-Whitney *U* test comparing the expression level by quantitative RT-PCR of patients with amplification of this gene determined by aCGH compared with those cases without (see Fig. 2). The CRA includes all genes that are amplified in more than 25 of 29 cases.
Abbreviations: nd, not determined; ns, not significant.

previously identified 11q13.3 genes, *MYEOV*, *CCND1*, *ORAOV1*, *FGF3*, *FGF4*, and *CTTN* (12–15), and three hypothetical genes (not further analyzed in this study; Fig. 1C). Of these 13 genes, 9 are coamplified in 27 of 29 cases (Table 1). The smallest overlapping region of amplification, found in 28 of 29 carcinomas, was restricted to a very small region of ~50 kb at position 69.7 Mb (NCBI build 35) and only contains a single gene, *FADD* (dark gray region in Fig. 1B and C; Table 1).

DNA amplification results in increased expression of numerous 11q13.3 genes. DNA amplification is generally accepted as a mechanism resulting in increased gene expression in drug resistance or carcinogenesis (4, 29). Based on these findings, we hypothesized that the key gene in the commonly amplified 11q13.3 region should not only be amplified most frequently but also be overexpressed when amplified. To identify the gene that fulfills this criterion the best, we designed a quantitative RT-PCR for all 13 genes (hypothetical genes were excluded) within the 1.7-Mb CRA. As a control, we also included three genes immediately centromeric of the CRA that were coamplified in only 9 of 29 cases (*CTP1a*, *MRPL21*, and *IGHMBP2*) as well as five genes on the telomeric side of the CRA that were not frequently (co-)amplified in our series of carcinomas but have been described to be overexpressed in other studies (*WNT-11*, *UVRAG*, *EMSY*, *GARP*, and *PAK1*). From 8 patients (Fig. 1: L03-L07 and P09-P11) and 10 without 11q13.3 amplification (A01-A10), total RNA was isolated from snap-frozen primary laryngeal carcinomas. To calculate the effect of DNA amplification on gene expression of each separate gene, the normalized ΔC_t ratios from the 10 patients without amplification were used as a calibrator (set at 1). *FGF3* and *FGF4* were excluded from further analysis because these genes were expressed in only 2 and none of the 18 carcinomas, respectively (not shown). Relative expression analysis per patient revealed that every patient with 11q13.3 amplification showed an increased expression for multiple amplified genes. To identify the gene with the best relation between amplification and expression, we compared the relative expression levels for each gene in the amplified and nonamplified groups by one-tailed Mann-Whitney *U* test (Fig. 2). This analysis revealed significant differences in gene expression for 8 of the 11 genes located in the CRA (Fig. 2A; Table 1). From these eight genes, the most significant relation ($P < 0.0001$) between increased expression and amplification was observed for *TPCN2*, *ORAOV1*, *PPFIA1*, and *FADD*. Correlating gene copy number to relative gene expression by linear regression analysis further confirmed this relation for these four genes (data not shown). Interestingly, high expression of *CPT1a*, *MRPL21*, and *IGHMBP2* was significantly correlated with amplification despite coamplification in only 8 of the 29 cases (Fig. 2B; Table 1). On the other hand, expression of *WNT-11*, *UVRAG*, *EMSY*, *GARP*, and *PAK1* (all located far outside the CRA) was not increased in carcinomas with amplification of the CRA (data not shown).

FADD is a new potential driver gene within the 11q13 amplicon in head and neck carcinoma. When combining the findings obtained by high-resolution aCGH mapping and expression analysis (summary given in Table 1), *FADD* was one of the most likely potential driver genes. Of the eight genes with a significant correlation between gene amplification and increased expression, *FADD* is amplified with the highest frequency (in 28 of 29 cases). To determine whether an increase in *FADD* mRNA due to DNA amplification also results in increased FADD protein expression, we carried out immunohistochemistry on formaldehyde-fixed/paraffin-embedded tissues from the same laryngeal carcinomas used for both RT-PCR and high-resolution aCGH analysis. We used an anti-FADD antibody that was previously used to detect total FADD expression on archive material (30). Normal epithelium present in most samples showed cytoplasmic staining of the suprabasal layer. In carcinoma cells, FADD protein expression was found mainly in the cytoplasm also and very homogeneously distributed in most tumors. In cases with the strongest expression, the FADD protein was not only detected in the cytoplasm but also nuclear staining became apparent. However, the staining intensity was variable between different carcinomas. Using the normal epithelium as a reference for normal expression levels, we categorized all samples as low FADD (FADD- and FADD+) and high FADD (FADD++ and FADD+++) expressing (Fig. 3). All 8 cases with high levels of *FADD* RNA also showed high FADD protein expression levels, whereas only 2 of 10 cases with low *FADD* RNA levels had elevated protein expression. Western blot analysis with the same anti-FADD antibody of lysates from frozen tissues of two cases with high and five cases with low FADD protein levels showed similar results (data not shown). Because RNA levels correlated significantly with DNA amplification and protein levels were significantly increased at high RNA expression, logistic regression analysis revealed a significant relation between amplification and protein expression ($P = 0.008$, Mann-Whitney *U* test). Because FADD is involved in regulation of apoptosis, we stained a subset of carcinomas for cleaved caspase-3 and found no correlation with FADD (not shown).

To investigate whether FADD expression is associated with clinicopathologic features, we determined expression levels in a series of 167 primary advanced-stage laryngeal carcinomas of patients who did not receive other therapy before surgery. High FADD levels were observed in 62 of 140 (44%) tested laryngeal cases. Univariate Cox regression analysis revealed that high FADD levels were related to an increased hazard ratio (HR) for worse overall survival [HR, 1.74; 95% confidence interval (95% CI), 1.07-2.83; $P = 0.025$] and disease-specific survival (HR, 3.29; 95% CI, 1.39-7.80; $P = 0.007$; Fig. 4A). Lymph node positivity (HR, 4.45; 95% CI, 1.94-10.20; $P < 0.001$) and radiotherapy treatment (HR, 3.42; 95% CI, 1.24-9.43; $P = 0.018$) also predicted a worse prognosis. A multivariate Cox regression model including FADD, lymph node metastasis, and treatment revealed that high FADD expression was significantly related to a worse prognosis (HR, 4.59; 95% CI, 1.56-13.47; $P = 0.004$). Furthermore, survival curves stratified for FADD expression and lymph node positivity showed that high FADD expression independent of the lymph node status identifies patients with poor prognosis (Fig. 4B).

Recently, nuclear localization of FADD has been ascribed to FADD phosphorylation at Ser^{194} and is important for its function in cell cycle control (31). Because the antibody against FADD used in this study does not discriminate between Ser^{194} phosphorylated (pFADD) and nonphosphorylated FADD, we also studied expression of pFADD with a specific anti-pFADD antibody. The immunohistochemical staining of pFADD was more heterogeneously distributed within tumor tissues compared with FADD and predominantly found within the nucleus. High pFADD levels were observed in 61 of 133 (46%) cases. Although FADD and pFADD stainings were

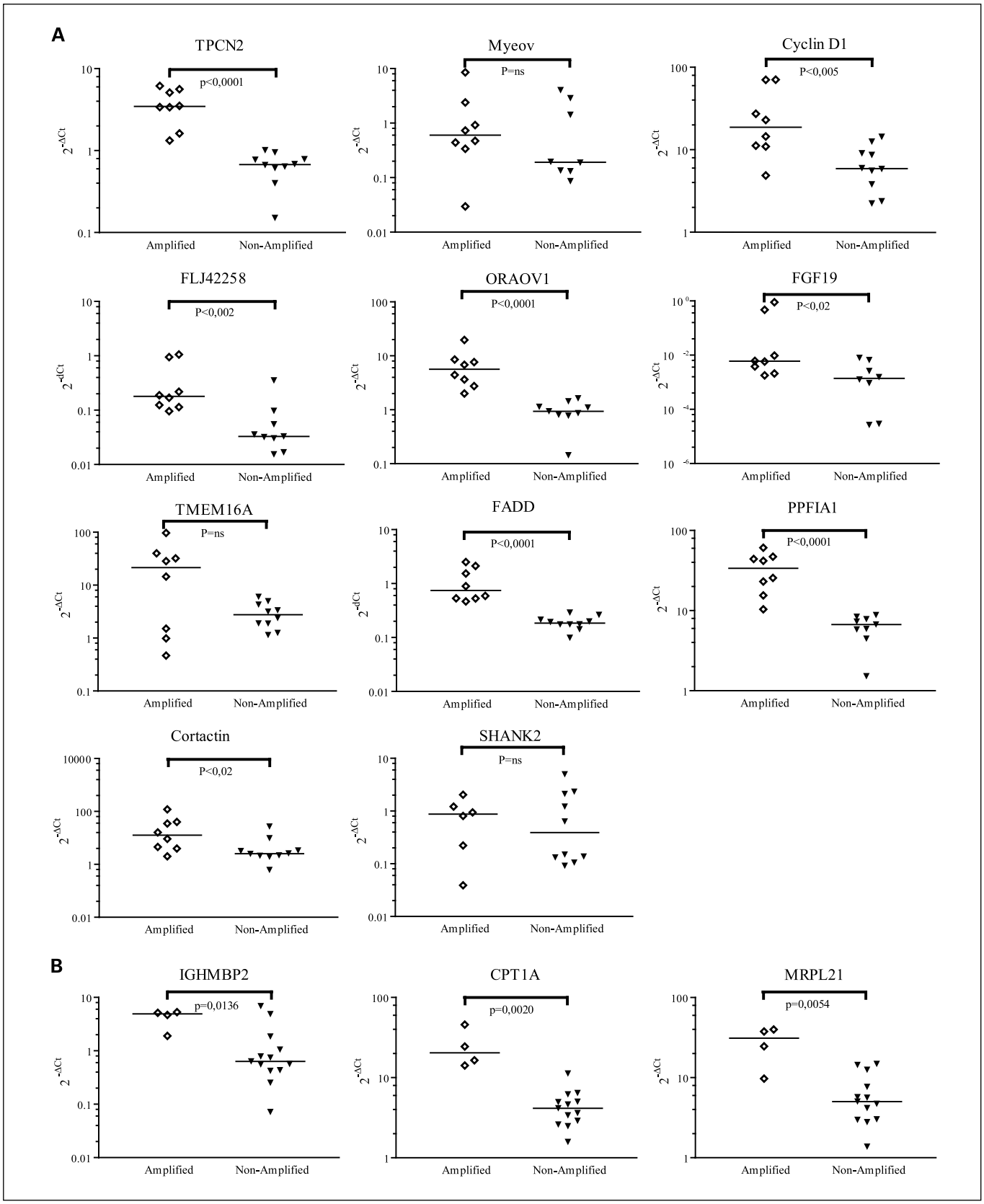

Fig. 2. Gene expression in 18 patients with or without amplification. *A,* expression of genes in the CRA. *B,* expression of genes near the CRA. Patients are divided into two groups based on the copy number status of the gene. Gene expression levels for each patient (◇, amplification; ▼, normal) are shown as $2^{-\Delta C_t}$ on the *y*-axis. The median expression is indicated per group by a horizontal line. Statistical analysis was done with a Mann-Whitney *U* test comparing gene expression for patients with amplification to those without.

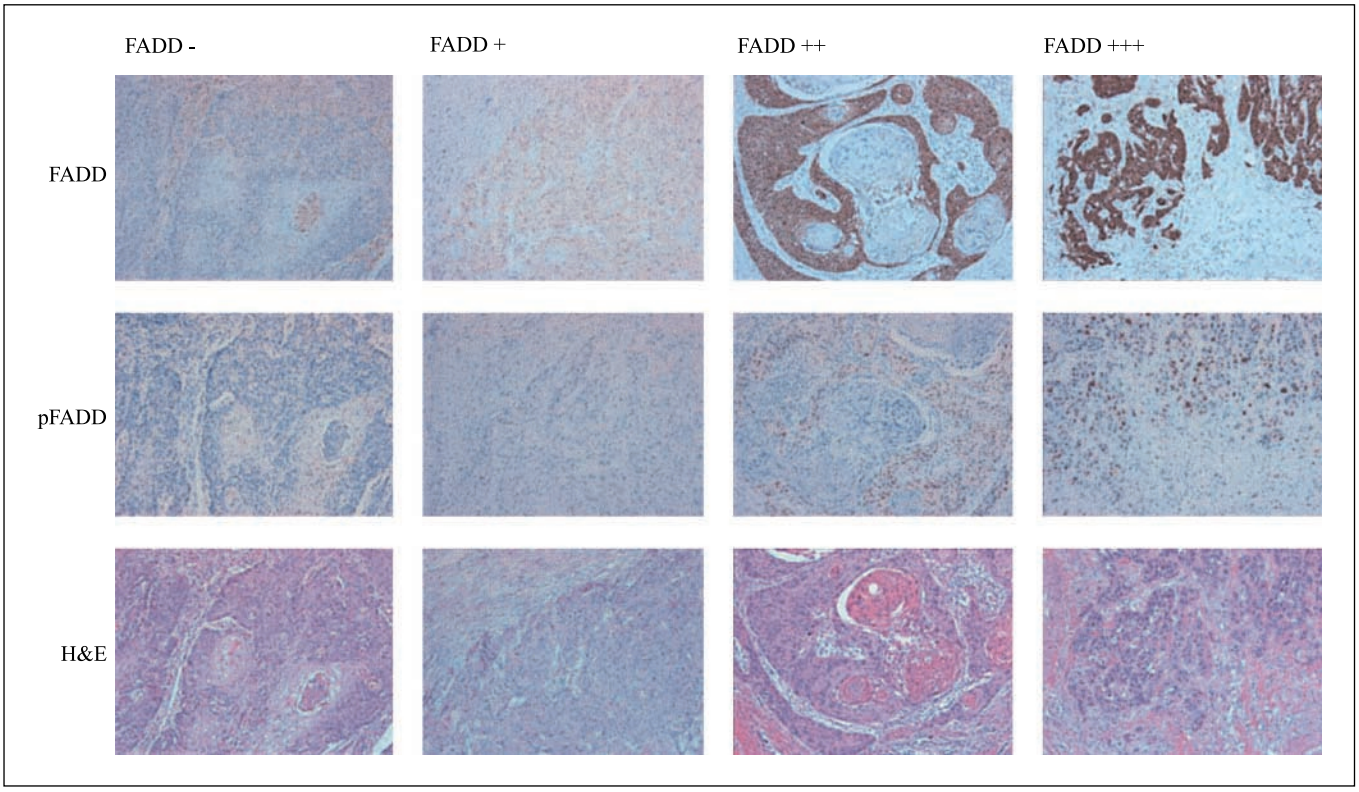

Fig. 3. Immunohistochemical comparison of FADD and pFADD expression. Protein expression for FADD and Ser^{194} phosphorylated FADD is shown on sequential sections for four patients. Patients are ordered from left to right by their relative FADD expression (FADD-, FADD+, FADD++, and FADD+++). FADD- and FADD+ are considered FADD Low and FADD++ and FADD+++ are considered FADD High in this study.

morphologically different, (logistic regression revealed a relation between the two stainings relative risk, 2.26; 95% CI, 1.11-4.59; $P = 0.025$). High expression of pFADD marked a worse overall survival (HR, 1.62; 95% CI, 0.98-2.68; $P = 0.061$) and disease-specific survival (HR, 3.05; 95% CI, 1.29-7.22; $P = 0.011$). When comparing patients with a low FADD to patients with a high expression of both protein isoforms, survival rates declined even further (Fig. 4C).

In summary, we show for the first time that amplification of the 11q13.3 region in head and neck cancer results in increased levels of the phosphorylated and nonphosphorylated FADD protein and that these increased levels are associated with a worse prognosis independent of the presence of lymph node metastasis.

Discussion

Chromosomal DNA amplification is a well-documented mechanism for cells to increase gene expression of a driver gene involved in oncogenesis, development, or multidrug resistance (4, 29). In many amplicons, the driver gene has yet to be identified. In search for such gene, any driver gene for amplification should fulfill at least two criteria: it should be most frequently amplified and it should have the most significant correlation between DNA amplification and expression levels. In the last 15 years, many efforts were undertaken to identify the driver gene in the 11q13 amplicon (6, 7) and numerous candidate genes were reported that showed increased expression on DNA amplification, including *CCND1* (12, 13), *CTTN* (13), *MYEOV* (14), *ORAOV1* (15), *EMSY* (16), and *SHANK2* (17). In this study, we carried out a comprehensive analysis of DNA amplification using high-resolution aCGH of the 11q13 region in combination with quantitative expression levels using quantitative RT-PCR of all genes in the 11q13 amplicon on a large series of laryngeal/pharyngeal carcinomas with 11q13 amplification ($n = 29$). The CRA contains 13 genes. Quantitative RT-PCR analysis revealed that not a single gene but eight genes showed overexpression on DNA amplification (Table 1).

In agreement with our findings, very recently, Järvinen et al. (32) proposed that *FADD* and *PPFIA1* are likely candidates, implicating that these two genes are located near the core of the amplicon. However, our comprehensive analysis of all genes in the 11q13 region revealed that, in addition to *FADD* and *PPFIA1*, six other likely candidate genes (*TPCN2*, *CCND1*, *FLJ42258*, *ORAOV1*, *FGF19*, and *CTTN*) within the 11q13.3 CRA are coamplified in at least 25 of 29 cases and show a strong correlation between expression and amplification (Table 1). Of the genes in the 11q13.3 amplicon, *CCND1* (12, 13) and *CTTN* (10, 18–20) were studied most extensively in the last 15 years. They have been reported to be the best candidates for driving 11q13.3 amplification because they are both frequently coamplified and amplification correlates well with overexpression. Furthermore, the characterization of the cell biological properties of both these genes provided clear evidence for a function in processes related to tumorigenesis such as cell cycle control and cell migration (18, 19, 33, 34). *ORAOV1* was described as a candidate in one study using quantitative copy

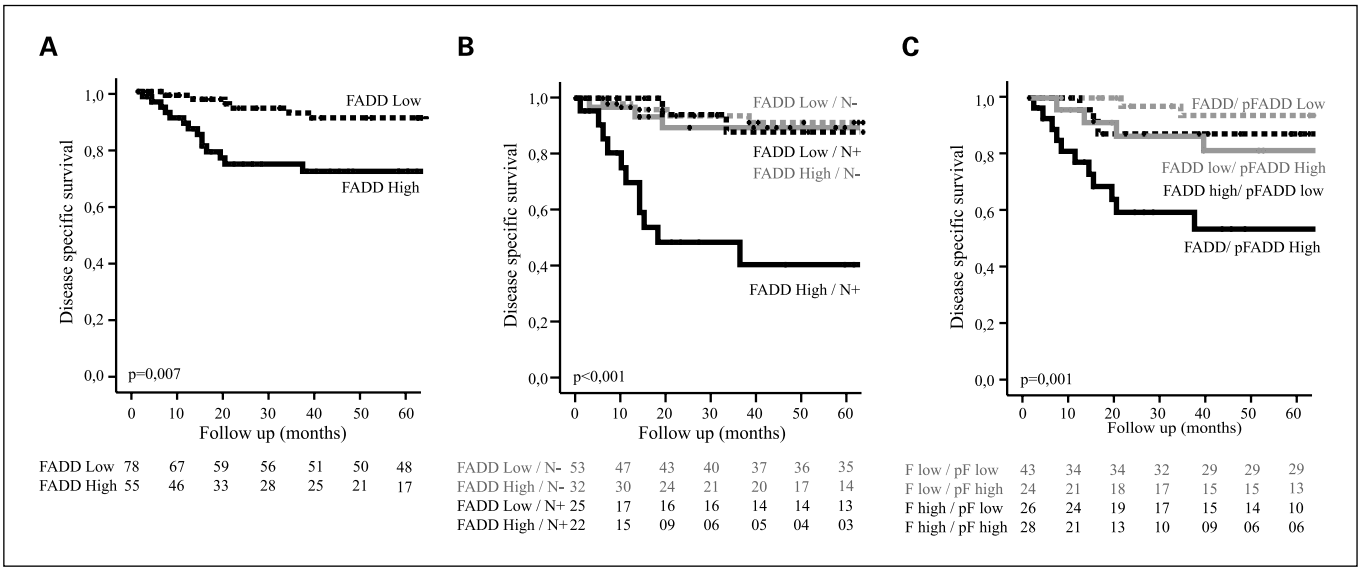

Fig. 4. Survival analysis related to FADD and pFADD expression. Kaplan-Meier analysis was done for disease-free survival. *A,* patients are shown as high or low FADD expressing. *B,* the survival of lymph node positive (*N+*) and lymph node negative (*N-*) tumors stratified for FADD expression. *C,* disease-specific survival for FADD stratified for Ser^{194} phosphorylated FADD expression. The number of patients remaining after 5 y is shown underneath the plots. Significance was calculated using univariate Cox analysis and *P* values are displayed on the bottom left of the plot.

number analysis (QuMA) with 11q13.3 markers in oral carcinoma cell lines (15). Here, we show that also in primary carcinomas of the head and neck *ORAOV1* seems a good candidate (Table 1; Fig. 2A), although the function of this gene is still unknown. *PPFIA1* is involved in regulating the disassembly of focal adhesions (35) but its function has not yet been studied in relation to 11q13.3 amplification. The functions of *TPCN2*, *FGF19*, and *FLJ42258* have not yet been studied in relation to 11q13.3 amplification.

A general increase in the expression of numerous genes within an amplicon, known as a gene dosage effect, has previously been shown by several groups for other amplified chromosomal loci (24, 36–38). Recently, we reported on the structural analysis of the amplified 11q13 region in the same 29 laryngeal carcinomas using a high-resolution 11q13-specific aCGH (25). We found that the boundaries of the commonly amplified region were restricted to four segments, coinciding with segmental duplications and syntenic breakpoints. These findings imply that the selection of genes in the 11q13.3 amplicon is determined by the ability to form DNA breaks within specific fragile regions and consequently would result in large amplicons containing multiple genes. Because of coincidental coamplification, the increased gene dosage will result in increased expression of genes that either act as driver or are not harmful. To identify those overexpressed gene(s) that are relevant to tumor progression, functional analysis of all the eight genes would be necessary. At present, the cell biological properties of CCND1 (33), CTTN (39), and FADD (ref. 40 and see below) have been reported. On the other hand, the existence of multiple driver genes cooperating at different time points in carcinogenesis or selected for at different stages of carcinogenesis is an attractive alternative when considering gene functions within in this amplicon. For instance, CCND1 might cause growth advantage and genetic instability at early stages (41); CTTN could enhance cell migration (18–20, 34); and FADD may be involved in survival and therapy resistance (see below). In this way, the same 11q13.3 amplicon is selected for at multiple stages of carcinogenesis.

FADD, a new driver within the amplified 11q13.3 region in head and neck cancer. In this study, *FADD* was identified as one of the eight potential driver genes in the 11q13 amplicon in head and neck cancer. In fact, *FADD* was amplified the highest (28 of 29) and its expression strongly correlated with DNA amplification. FADD was originally identified as a protein that binds to the cytosolic tail of the FAS receptor (reviewed in ref. 42). The proapoptotic adapter molecule recruits caspase-8 and caspase-10 to initiate the formation of the death-inducing signal complex that mediates receptor-induced apoptosis. The recruitment of these caspases to the death-inducing signal complex leads to intracellular processing and activation of caspases, eventually resulting in cleavage of downstream targets and apoptosis. Thus, increased expression should correlate with increased apoptosis. However, more recent studies showed that FADD also plays an important role in growth and regulation of the cell cycle (reviewed in ref. 43). In both FADD-knockout and transgenic mice expressing human dominant-negative FADD, T cells are defective in proper cell cycle entry in response to mitogens (44). *In vitro* experiments showed that FADD was regulated during cell cycle progression. Cells treated with agents blocking the G_2-M transition have higher levels of Ser^{194} phospho-FADD (45). Furthermore, expressing a Ser^{194} phospho-mimicking FADD mutant caused G_2-M cell cycle arrest (46), suggesting a key role in cell cycle regulation (40, 46–48).

Chen et al. (30) showed that increased expression of FADD and Ser^{194} phosphorylated FADD were both associated with decreased survival of patients with lung adenocarcinoma. In contrast to head and neck carcinomas, in these lung adenocarcinomas DNA amplification of *FADD* was not detected (30). Furthermore, patients with laryngeal/pharyngeal carcinomas with high levels of FADD and pFADD have a significantly decreased survival (Fig. 4C). In patients with carcinomas

containing high FADD expression and lymph node metastases, the prognosis is even worse (Fig. 4B). This indicates that high FADD expression defines a worse prognosis for patients independent of other classic predictors such as lymph node status. Interestingly, in lung adenocarcinoma, the staining of pFADD was correlated with Ki-67, cyclin B1, and CCND1 staining, indicating that high pFADD levels are associated with increased cell proliferation at the G_2-M phase of the cell cycle (30). This is in concordance with *in vitro* studies (30, 31, 45, 47, 49). Because this amplification is prominent in head and neck carcinoma (6) and results in increased expression of FADD (this study), FADD might be one of the genes to regulate cell cycle progression in laryngeal carcinomas with 11q13 amplification.

Future perspectives on the role of FADD and chemotherapeutic drugs in head and neck cancer. The role of FADD in cell cycle regulation suggests that FADD is also implicated in the response to cytotoxic drugs. As previously described, Taxol treatment arrests tumor cells at G_2-M with concomitant phosphorylation of FADD (47). Interestingly, both the G_2-M arrest and cell growth suppression on Taxol treatment were abolished in transfected cells expressing a non–phosphomimicking mutant of FADD (31, 40, 48). Taken together, these data show that cells expressing high levels of phospho-FADD are (30) more sensitive to Taxol-induced cell cycle arrest (31, 40) than cells expressing nonphosphorylated FADD. Our data revealed that in HNSCC carcinomas with 11q13.3 amplification, both FADD and pFADD are overexpressed (this work). Several recent clinical studies in HNSCC have underlined a positive effect of chemoradiation over radiotherapy alone (3, 50). We hypothesize that patients with HNSCC carcinomas with 11q13.3 amplification and concomitant pFADD overexpression might benefit from Taxol-based chemoradiotherapy over radiotherapy alone.

Acknowledgments

We thank P. van der Vlies and T. Dijkhuizen (University Medical Center Groningen), J.A. Veltman and E.F. Schoenmakers (Radboud University Nijmegen Medical Center, Nijmegen), and F. Balm (the Netherlands Cancer Institute-Antoni Van Leeuwenhoek Hospital, Amsterdam).

References

1. Nix PA, Greenman J, Cawkwell L, Stafford ND. Defining the criteria for radioresistant laryngeal cancer. Clin Otolaryngol Allied Sci 2004;29:705–8.
2. Bernier J, Cooper JS. Chemoradiation after surgery for high-risk head and neck cancer patients: how strong is the evidence? Oncologist 2005;10:215–24.
3. Forastiere AA, Goepfert H, Maor M, et al. Concurrent chemotherapy and radiotherapy for organ preservation in advanced laryngeal cancer. N Engl J Med 2003;349:2091–8.
4. Albertson DG. Gene amplification in cancer. Trends Genet 2006;22:447–55.
5. Morrison C, Zanagnolo V, Ramirez N, et al. HER-2 is an independent prognostic factor in endometrial cancer: association with outcome in a large cohort of surgically staged patients. J Clin Oncol 2006;24:2376–85.
6. Schuuring E. The involvement of the chromosome 11q13 region in human malignancies: cyclin D1 and EMS1 are two new candidate oncogenes – a review. Gene 1995;159:83–96.
7. Ormandy CJ, Musgrove EA, Hui R, Daly RJ, Sutherland RL. Cyclin D1, EMS1 and 11q13 amplification in breast cancer. Breast Cancer Res Treat 2003;78:323–35.
8. Akervall JA, Jin Y, Wennerberg JP, et al. Chromosomal abnormalities involving 11q13 are associated with poor prognosis in patients with squamous cell carcinoma of the head and neck. Cancer 1995;76:853–9.
9. Hermsen M, Alonso GM, Meijer G, et al. Chromosomal changes in relation to clinical outcome in larynx and pharynx squamous cell carcinoma. Cell Oncol 2005;27:191–8.
10. Rodrigo JP, Garcia LA, Ramos S, Lazo PS, Suarez C. EMS1 gene amplification correlates with poor prognosis in squamous cell carcinomas of the head and neck. Clin Cancer Res 2000;6:3177–82.
11. Williams ME, Gaffey MJ, Weiss LM, Wilczynski SP, Schuuring E, Levine PA. Chromosome 11Q13 amplification in head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 1993;119:1238–43.
12. Lammie GA, Fantl V, Smith R, et al. D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1. Oncogene 1991;6:439–44.
13. Schuuring E, Verhoeven E, Mooi WJ, Michalides RJ. Identification and cloning of two overexpressed genes, U21B31/PRAD1 and EMS1, within the amplified chromosome 11q13 region in human carcinomas. Oncogene 1992;7:355–61.
14. Janssen JW, Cuny M, Orsetti B, et al. MYEOV: a candidate gene for DNA amplification events occurring centromeric to CCND1 in breast cancer. Int J Cancer 2002;102:608–14.
15. Huang X, Gollin SM, Raja S, Godfrey TE. High-resolution mapping of the 11q13 amplicon and identification of a gene, TAOS1, that is amplified and overexpressed in oral cancer cells. Proc Natl Acad Sci U S A 2002;99:11369–74.
16. Hughes-Davies L, Huntsman D, Ruas M, et al. EMSY links the BRCA2 pathway to sporadic breast and ovarian cancer. Cell 2003;115:523–35.
17. Freier K, Sticht C, Hofele C, et al. Recurrent coamplification of cytoskeleton-associated genes EMS1 and SHANK2 with CCND1 in oral squamous cell carcinoma. Genes Chromosomes Cancer 2006;45:118–25.
18. Luo ML, Shen XM, Zhang Y, et al. Amplification and overexpression of CTTN (EMS1) contribute to the metastasis of esophageal squamous cell carcinoma by promoting cell migration and anoikis resistance. Cancer Res 2006;66:11690–9.
19. van Rossum AG, Moolenaar WH, Schuuring E. Cortactin affects cell migration by regulating intercellular adhesion and cell spreading. Exp Cell Res 2006;312:1658–70.
20. Weed SA, Parsons JT. Cortactin: coupling membrane dynamics to cortical actin assembly. Oncogene 2001;20:6418–34.
21. Pinkel D, Segraves R, Sudar D, et al. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat Genet 1998;20:207–11.
22. Veltman JA, Schoenmakers EF, Eussen BH, et al. High-throughput analysis of subtelomeric chromosome rearrangements by use of array-based comparative genomic hybridization. Am J Hum Genet 2002;70:1269–76.
23. Albertson DG, Ylstra B, Segraves R, et al. Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. Nat Genet 2000;25:144–6.
24. Watson JE, Doggett NA, Albertson DG, et al. Integration of high-resolution array comparative genomic hybridization analysis of chromosome 16q with expression array data refines common regions of loss at 16q23-qter and identifies underlying candidate tumor suppressor genes in prostate cancer. Oncogene 2004;23:3487–94.
25. Gibcus JH, Kok K, Menkema L, et al. High-resolution mapping identifies a commonly amplified 11q13.3 region containing multiple genes flanked by segmental duplications. Hum Genet 2007;121:187–201.
26. Hermsen M, Guervos MA, Meijer G, et al. New chromosomal regions with high-level amplifications in squamous cell carcinomas of the larynx and pharynx, identified by comparative genomic hybridization. J Pathol 2001;194:177–82.
27. Takes RP, Baatenburg de Jong RJ, Schuuring E, et al. Markers for assessment of nodal metastasis in laryngeal carcinoma. Arch Otolaryngol Head Neck Surg 1997;123:412–9.
28. Imoto I, Yang ZQ, Pimkhaokham A, et al. Identification of cIAP1 as a candidate target gene within an amplicon at 11q22 in esophageal squamous cell carcinomas. Cancer Res 2001;61:6629–34.
29. Stark GR, Debatisse M, Giulotto E, Wahl GM. Recent progress in understanding mechanisms of mammalian DNA amplification. Cell 1989;57:901–8.
30. Chen G, Bhojani MS, Heaford AC, et al. Phosphorylated FADD induces NF-κB, perturbs cell cycle, and is associated with poor outcome in lung adenocarcinomas. Proc Natl Acad Sci U S A 2005;102:12507–12.
31. Bhojani MS, Chen G, Ross BD, Beer DG, Rehemtulla A. Nuclear localized phosphorylated FADD induces cell proliferation and is associated with aggressive lung cancer. Cell Cycle 2005;4:1478–81.
32. Jarvinen AK, Autio R, Haapa-Paananen S, et al. Identification of target genes in laryngeal squamous cell carcinoma by high-resolution copy number and gene expression microarray analyses. Oncogene 2006;25:6997–7008.
33. Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A, Schmidt EV. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature 1994;369:669–71.
34. van Rossum AG, de Graaf JH, Schuuring-Scholtes E, et al. Alternative splicing of the actin binding domain of human cortactin affects cell migration. J Biol Chem 2003;278:45672–9.
35. Serra-Pages C, Kedersha NL, Fazikas L, Medley Q, Debant A, Streuli M. The LAR transmembrane protein tyrosine phosphatase and a coiled-coil LAR-interacting protein co-localize at focal adhesions. EMBO J 1995;14:2827–38.
36. Heidenblad M, Lindgren D, Veltman JA, et al.

Microarray analyses reveal strong influence of DNA copy number alterations on the transcriptional patterns in pancreatic cancer: implications for the interpretation of genomic implications. Oncogene 2005;24:1794–801.

37. Monni O, Barlund M, Mousses S, et al. Comprehensive copy number and gene expression profiling of the 17q23 amplicon in human breast cancer. Proc Natl Acad Sci U S A 2001;98:5711–6.

38. Cromer A, Carles A, Millon R, et al. Identification of genes associated with tumorigenesis and metastatic potential of hypopharyngeal cancer by microarray analysis. Oncogene 2004;23:2484–98.

39. Patel AS, Schechter GL, Wasilenko WJ, Somers KD. Overexpression of EMS1/cortactin in NIH3T3 fibroblasts causes increased cell motility and invasion *in vitro*. Oncogene 1998;16:3227–32.

40. Alappat EC, Volkland J, Peter ME. Cell cycle effects by C-FADD depend on its C-terminal phosphorylation site. J Biol Chem 2003;278:41585–8.

41. Izzo JG, Papadimitrakopoulou VA, Li XQ, et al. Dysregulated cyclin D1 expression early in head and neck tumorigenesis: *in vivo* evidence for an association with subsequent gene amplification. Oncogene 1998; 17:2313–22.

42. Tibbetts MD, Zheng L, Lenardo MJ. The death effector domain protein family: regulators of cellular homeostasis. Nat Immunol 2003;4:404–9.

43. Park SM, Schickel R, Peter ME. Nonapoptotic functions of FADD-binding death receptors and their signaling molecules. Curr Opin Cell Biol 2005;17: 610–6.

44. Osborn SL, Sohn SJ, Winoto A. Constitutive phosphorylation mutation in FADD results in early cell cycle defects. J Biol Chem 2007;282:22786–92.

45. Shimada K, Matsuyoshi S, Nakamura M, Ishida E, Kishi M, Konishi N. Phosphorylation of FADD is critical for sensitivity to anticancer drug-induced apoptosis. Carcinogenesis 2004;25:1089–97.

46. Gomez-Angelats M, Cidlowski JA. Molecular evidence for the nuclear localization of FADD. Cell Death Differ 2003;10:791–7.

47. Alappat EC, Feig C, Boyerinas B, et al. Phosphorylation of FADD at serine 194 by CKIα regulates its nonapoptotic activities. Mol Cell 2005;19:321–32.

48. Matsuyoshi S, Shimada K, Nakamura M, Ishida E, Konishi N. FADD phosphorylation is critical for cell cycle regulation in breast cancer cells. Br J Cancer 2006;94:532–9.

49. Scaffidi C, Volkland J, Blomberg I, Hoffmann I, Krammer PH, Peter ME. Phosphorylation of FADD/MORT1 at serine 194 and association with a 70-kDa cell cycle-regulated protein kinase. J Immunol 2000; 164:1236–42.

50. Gupta N, Hu LJ, Deen DF. Cytotoxicity and cell-cycle effects of paclitaxel when used as a single agent and in combination with ionizing radiation. Int J Radiat Oncol Biol Phys 1997;37:885–95.